THE INFLUENCE OF CERTAIN COLLOIDS UPON FERMENTATION. Part ii.

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It was shown in Part i of this series that certain mineral and other colloids accelerated the fermentation of citrate and of the slowly fermentable carbohydrate, lactose, by the high temperature organism. The work in the main was in agreement with results obtained by investigators who had tested other bacteria and the case seems clear so far as the bacteria are concerned. It, however, seems peculiar that other living organisms such as yeasts are not affected in the same way. Söhngen found that while colloids generally had no influence upon the activity of yeast, the bio-colloids, such as peat, paper, charcoal and soil, hastened the alcoholic fermentation by assisting the elimination of the carbon dioxide from the fermenting fluid. He used five grams of pressed yeast with 100 c.c. of 10 per cent. dextrose and it is perhaps to be expected that the dispersal of the carbon dioxide would be advantageous in a rapid fermentation such as this would be.

As it did not seem reasonable to expect that the mineral colloids would really differ in their behaviour towards bacteria and yeasts, a few tests were made to see how far Söhngen's conclusions were justified. The apparatus used for determining the production of carbon dioxide by the high temperature organism was still in commission and it was used in some tests for ascertaining the influence of a colloid upon the fermentation of dextrose by yeast. The results, however, were not sufficiently consistent to enable any deduction to be made.

If we assume that the colloid acts by adsorbing the cellular enzymes and also the fermentable substrate, it is easy to understand that they would have no influence upon the endo-cellular enzyme, zymase. Yeasts, however, possess some extra-cellular enzymes and one of them is invertase. It is possible that if yeast were grown with sucrose, the colloid might hasten the activity of the invertase and the larger amount of the resulting invert sugar would give rise to an increased production of carbon dioxide.

The matter was tested and two small experiments, one with silica, the other with asbestos, were in favour of the idea. A third test may be given *in extenso*. It was made with 0.5 gram of saccharose and 0.2 gram of activated silica in 30 c.c. of dilute yeast water containing 0.2% of potassium phosphate and 0.1% of magnesium sulphate. The pH value was brought to 6.2. A few drops of a well distributed suspension of distillery yeast were used, the same number of drops being added to each flask.

The experiment showed that silica brought about an increased production of carbon dioxide upon the second and third days. It had apparently assisted the yeast invertase to invert the sucrose. In its way, this result with yeast is in agreement with the previous experiments with the high temperature organism.

Table i.—The Influence of Silica upon the Inversion of Saccharose and subsequent Fermentation of the Invert-sugar by Yeast.

Daily yields of carbon dioxide in mg. at 24°.

Days	1	2	3	4	5
Silica, 0.2 gram	54	131	67	12	2
	54	129	71	11	2
	55	127	60	18	2
Average	54	129	66	14	2
Control (no Colloid) ..	58	111	57	17	9
	54	110	51	31	14
	55	110	57	31	7
Average	56	111	55	26	10

The effect of a colloid upon the inversion of saccharose was then tested directly by noting the disappearance of saccharose. Two flasks each with 450 c.c. of nutritive fluid containing 20% of saccharose were seeded each with three drops of a well distributed suspension of a distillery yeast. One flask received 0.5% of asbestos as the colloid. By a mistake the flasks were incubated at 38° during the first day instead of 30° as was intended, but as the inversion appeared to be progressing favourably, the flasks were maintained at the higher temperature. The sugars were estimated according to the routine followed by Lane and Eynon (*Journ. Soc. Chem. Ind.* 1923, 32T.), the only modification being in the use of a flask provided with a cork pierced with three holes, one for the nozzle of the burette, one for introducing the methylene blue, and the third holding an angular tube as a steam get-away.

Table ii.—The Influence of Asbestos on the Inversion of Saccharose by Yeast at 38°.

Days	Start	1	2	3
Asbestos, saccharose inverted	—	8.3	13.8	14.2
saccharose not inverted ..	20	11.3	3.0	0.5
Control, saccharose inverted	—	6.2	10.3	10.3
saccharose not inverted ..	20	13.3	6.5	3.8

The rate of disappearance of the saccharose shows that the colloid had a decided influence in accelerating the inversion activity of the yeast.

A confirmative test was made with silica as the colloid and the temperature was maintained at 30° instead of at 38° as in the last case.

The influence of the colloid is not so clearly shown in this as in the last experiment. It may be the difference of the colloid but it is more likely the effect of the difference in the temperature. Invertase has its optimum working temperature between 55° and 60°, and it is to be expected that the nearer the fermentation temperature is to this the greater will be the inversion.

Table iii.—The Influence of Silica on the Inversion of Saccharose by Yeast at 30°.

Days	Start	1	2	3	4	5	6
Silica, saccharose inverted ..	—	1.3	4.0	8.7	10.4	11.2	10.3
saccharose not inverted ..	20.2	18.3	—	8.8	5.2	3.4	1.2
saccharose unaccounted for	—	—	—	2.7	4.6	5.6	8.7
Control, saccharose inverted ..	—	1.2	3.8	8.2	10.0	10.9	10.5
saccharose not inverted ..	20.2	18.9	—	9.5	6.1	4.0	1.6
saccharose unaccounted for	—	—	—	2.5	4.1	5.3	8.1

The last two experiments confirm the previous finding that colloids such as asbestos and silica, accelerate the activity of organisms which secrete extracellular enzymes such as invertase.

In summing up the last table and noting the saccharose unaccounted for, presumably to form alcohol, carbon dioxide, glycerin and fixed acids, it would seem that the colloid did to a certain extent increase the production of alcohol and carbon dioxide.

As the point was of some importance, it was decided to test the matter by the disappearance of dextrose during its fermentation in the presence and absence of a colloid.

A test with the same nutritive fluid blended with glucose-syrup instead of saccharose showed a slightly greater disappearance of the dextrose from the third to the fifth day. Although less sugar disappeared from the control fluid, it seemed to be fermenting more freely. The foam on its surface was more persistent and when filtered to eliminate the carbon dioxide it was always more cloudy than the test fluid. Possibly the yeast cells had agglomerated on the particles of silica, but such could not be demonstrated microscopically nor was there any pronounced agglutination of the yeast cells in either of the fluids.

It seemed that the method of conducting the last experiment could be improved upon. The fermentation of the control fluid was apparently different from that of the test with the colloid and it seemed possible, in spite of a vigorous shaking just before drawing off the portions, that more yeast cells might have been abstracted from the control than from the test.

In the next experiment, the medium was divided into 50 c.c. portions contained in 100 c.c. conical flasks and whole portions were taken for the dextrose determination. The medium used was the same as the last, with the addition of 0.25% peptone, that is to say it contained starch-glucose syrup 360 grams, yeast water 100 c.c., monopotassium phosphate 2, anhydrous magnesium sulphate 1 and peptone 2.5 grams per litre. The pH value was brought to 6.0. The progeny from a single cell of a recently obtained pressed yeast was distributed in Hansen's fluid and six drops were added to each flask.

Table iv.—The Influence of Silica upon the Fermentation of Dextrose. Percentage of dextrose consumed.

Days	2	3	4	5	7
Silica, 0.25%..	47	53	55	58	68
Control	43	50	51	53	58

There was an increased consumption of the dextrose under the influence of the silica and it was maintained throughout the experiment.

The controls always had more foam on the surfaces of the fluids and this led to testing the viscosity of a fermenting solution containing starch-glucose. There was a slightly increased viscosity in the test containing silica but in view of the fact that the starch-glucose contained much dextrin, the experiment was repeated at a later date when fuller's earth was used as the colloid and commercial dextrose, containing 75% of dextrose, as the sugar. The medium was the same as that used in experiment iv, with the substitution of 16% of commercial dextrose for the starch glucose. Two flasks were used, each with 500 c.c. of medium; one received in addition 2.5 grams of fuller's earth. The seeding was a five days' culture at 28° of a distillery yeast in Hansen's fluid. The supernatant liquid had been decanted and replaced with diluted meat extract; the cells were broken apart and uniformly distributed by repeated passage through a capillary nozzle. Eight drops (about 0.25 c.c.) were added to each flask.

During fermentation, the control fluid carried more froth than the test and foamed strongly on being shaken; the test carried little froth and foamed slightly. On the second day, the test contained 7.08% of dextrose and the experiment was concluded on the third day. The fluids were filtered through paper, then through paper pulp in order to obtain brilliant solutions.

Table v.—The Influence of Fuller's Earth upon the Viscosity and Alcoholic Fermentation of Dextrose. Three days at 28°.

	Control	Fuller's earth
At start—		
Dextrose	11.75	11.75
At end—		
Dextrose	6.01	2.83
= Dextrose consumed %	49	76
Alcohol by weight	2.64	4.13
Viscosity (water = 1)	1.246	1.257

The viscosity of the test fluid was greater than that of the control, but as there was a difference of only ten seconds in the run of 18½ minutes by the 100 c.c. of liquid through the capillary nozzle, the difference is too small to explain any retention of carbon dioxide during fermentation. The difference in the dextrose consumed is the greatest that has been noted in these experiments with yeast. It was possibly due to the seeding cells being five days old and in consequence perhaps slightly enfeebled. The stimulating influence of the colloids upon enfeebled bacterial cells has been already noted.

It has been seen that silica hastened the fermentation of dextrose and it remained to show that the other insoluble colloids did the same.

The medium used was much the same as in the previous experiments and consisted of commercial glucose 100 grams, yeast water 100 c.c., potassium dihydrogen phosphate 5 grams, magnesium sulphate anhyd. 1 gram, and peptone 2 grams per litre; the reaction was brought to pH 6.0. The fluid when prepared contained 7.5% of dextrose. Three hundred c.c. portions were pipetted into a number of 500 c.c. flasks and with the exceptions of the agar and the controls each received 1.5 grams of colloid. Ten drops of a uniformly distributed suspension

of a distillery yeast were added to each flask. Set 1 was incubated at 37° but, as the fermentation seemed to have been too quick and it was hoped to obtain wider differences with a slower fermentation, the flasks of set 2 were incubated at 28°.

Table vi.—The Fermentation of Dextrose by Yeast. Percentage of dextrose consumed.

Set 1 at 37°			Set 2 at 28°		
Days	1	2	Days	2	3
Talc, 0.5%	23.9	80.7	Kieselguhr, 0.5%	67.9	91.2
Kaolin, 0.5%	20.9	80.6	French chalk, 0.5% ..	59.7	90.5
Asbestos, 0.5%	19.5	76.3	Agar, 0.2%	47.0	90.0
Fuller's earth, 0.5% ..	22.9	85.1	Control (no colloid) ..	53.3	87.9
Control (no colloid) ..	21.5	72.0			

All the colloids increased the consumption of dextrose, that is, they had hastened the fermentation by one or the other day of observation.

Set 2 incubated at 28° certainly showed greater differences than set 1 at 37°, probably because the fermentation was caught when from 50 to 60% of the dextrose had been fermented.

It was noted that the control of set 1 at the end of the first day contained more carbon dioxide in solution than the others as was evidenced by a more vigorous effervescence when the liquid was discharged upon a filter to eliminate the dissolved gas previous to measuring off the portion for analysis. This would make it appear that Söhngen's idea of the colloids increasing the fermentation by assisting the elimination of the carbon dioxide was correct. The asbestos test with the smaller consumption of dextrose was anomalous. It had a lessened effervescence like the other colloids, but the asbestos fibres had formed a felt-like film on the surface of the fermenting fluid and this had possibly prevented the aeration of the liquid. In set 2 the control test and the agar test, which was viscous, showed most effervescence upon filtration.

A few more colloidal substances were tested in a further experiment in which the peptone was replaced by marmite. The colloids undoubtedly accelerated, although the differences were not so pronounced as in the last table. This might have been caused by the substitution of marmite for the peptone.

Table via.—The Fermentation of Dextrose by Yeast. Percentage of dextrose consumed.

Set 1 at 28°					Set 2 at 37°			
Days	1	2	3	4	Days	1	2	3
Silicic acid, 0.05%	31.9*	50.5*	71.0*	91.7	Wood charcoal, 0.25%	40.3	70.3	91.0
Ferric hydroxide, 0.015%	28.9	52.6	75.8	92.6	Kieselguhr, 0.25% ..	37.5	70.4	91.7
Agar, 0.1%	31.2*	52.6	77.3	93.2	Talc, 0.25%	38.7	69.4	91.1
Fuller's earth, 0.25%	27.9	51.7	78.2	92.4	Gelatin, 0.25%	30.8*	63.4*	84.4*
Control (no colloid)	31.1*	50.5*	69.5*	90.0	Control (no colloid)..	38.1*	65.1*	86.2

The colloidal silicic acid and the colloidal ferric hydroxide were sterilized separately and added to the flasks previous to seeding. The hydroxide was

coagulated at once and the silicic acid separated out during the first day; they acted, therefore, as insoluble colloids.

The tests marked with an asterisk foamed when pipetted on to the filter, the others were still. The foaming does not seem to have much significance, for the first day's results are just the opposite of what should have happened if an excessive amount of carbon dioxide dissolved in the fluid had hindered the fermentation. On the other hand, in set 2 the gelatin test always effervesced when filtered and the amount of dextrose consumed was always lower than the control. So far the evidence is rather in favour of Söhngen's idea.

Agar which tended to hinder the fermentation when present to the extent of 0.2%, accelerated when a smaller quantity was added; the fluid was not so viscous. It is, however, irregular in its action. In another experiment in which 0.05% was used, it acted as a depressant. Its behaviour is under investigation.

Taken as a whole it is clear that certain mineral and other suspended colloids accelerate the activity of yeast. The substances that have been tested with positive results include silica, asbestos, ferric hydrate, talc, kaolin, fuller's earth, kieselguhr, French chalk, agar and charcoal.

The main point that has been brought out is that yeast, like the high temperature bacterium, ferments more actively in the presence of certain suspended colloids. It is true that the effects were not so great, but they were sufficiently pronounced to show that living bacteria and yeasts do not materially differ in this respect.

In my previous work with colloids, use had been made of the high temperature organism and the complete dissolution of a sugar to carbon dioxide had been taken as evidence of the bacterial activity. The work in this paper has shown that a partial dissolution, the inversion of saccharose by yeast, was increased by colloids. Some work that had been done with invertase itself necessitated a confirmation of the accelerating influence of colloids upon inversion by another living micro-organism.

The Inversion of Saccharose by Bac. levaniformans.

The organism chosen was *Bac. levaniformans*, an ally of *Bac. vulgatus*, occurring in sugar crystals in an almost pure condition. There are several races of this organism. One of them, previously described as $\beta\beta$ (Proc. Linn. Soc. N. S. Wales, 1901, 613), was isolated from brewers' crystals.

At the time of their isolation, small crystals of household sugar were examined and found to contain the normal white race. The race $\beta\beta$ grew as a yellow expansion on ordinary nutrient agar and gave no film on bouillon.

A heaped loop of an agar-culture was added to a bouillon-culture and the cells distributed by repeated passage through the capillary nozzle of a pipette. Four drops of the suspension were added to flasks containing 50 c.c. portions of a saccharose medium which consisted of saccharose 80, sodium phosphate 2, potassium chloride 5 and peptone 2 grams in a litre of tap-water. It had been brought to a pH value of 6.8 with dilute hydrochloric acid and had been steamed on six successive days.

After the flasks had been incubated at 37° for a day, it was noted that two of the controls were clear, while the others were turbid. This was again noted in a second experiment and also that some of the controls which had begun to ferment, had more gum than the others indicating that, although they had been seeded with the same number of cells of the same vitality, the onset of fermen-

tation in the control flasks had been irregular. This destroyed the scheme of the experiments. A similar irregularity had been noted in the case of the high temperature organism and had led to the examination of the influence of colloids upon fermentation.

The experience with the tests indicated that the seeding cells should have previously been grown in the same fluid, and also that the gum in the cultures should be eliminated by precipitation with alcohol when preparing the solution for the determination of the sugar. The method adopted was to pipette 25 c.c. of the fermented fluid into a 100 c.c. flask, neutralize it to phenolphthalein and make to volume with strong spirit. After standing overnight, the solution was decanted or filtered off and the alcohol eliminated by evaporation from an aliquot portion. The residual fluid was transferred to a 100 c.c. flask, slightly acidified to methyl-red with acetic acid, treated with lead subacetate, made to volume and filtered into a 100 c.c. graduated jar. After measuring, the filtrate was acidified with dilute acetic acid, treated with an excess of potassium oxalate and filtered. The sugar was determined in the filtrate.

Two experiments were made and the following results were obtained on the second day in each case. By the fourth day the amounts were very much the same at about 80% and are not recorded.

Table vii.—The Inversion of Saccharose by *Bac. levaniformans* in presence of some Colloids. Percentage of saccharose inverted in two days at 37°.

Set 1.		Set 2.	
Fuller's earth	69.0	Talc	58.4
Asbestos	64.7	Kaolin	47.5
Kieselguhr	56.6	Animal charcoal	40.0
French chalk	54.5	Willow charcoal	21.2
Silica	52.4	Control (no colloid)	31.7
Control (no colloid)	48.2		

All these colloids with the exception of willow charcoal accelerated the inversion of sugar. In a future paper I shall show that this form of charcoal does hasten the inversion and is no exception to the fact which seems to be well demonstrated that the mineral and other colloids such as have been tested, accelerate the inversion activity of *Bac. levaniformans.*

Bac. fluorescens liquefaciens is supposed to be able to secrete invertase, but when it was grown in a fluid containing sugar, no inversion of the saccharose occurred.

So far it has been shown that the mineral and other insoluble colloids accelerate the fermentation of dextrose by yeast, the inversion of saccharose by yeast and the inversion of saccharose by *Bac. levaniformans.* It seems to be a stimulation of the cell activity rather than an adsorption of ferment and fermentable substrate upon the colloidal surfaces. To test the matter it was decided to test the activity of a naked enzyme when in contact with the same colloids. The enzyme chosen was the same that had already been under investigation, viz., invertase.

The Action of Certain Colloids upon Invertase.

Invertase was prepared from autolysed yeast in the manner usually recommended and its activity was tested.

One-tenth of a gram of the dry powder was stirred with 10 c.c. of water which was acidified with oxalic acid to a pH value of 4.4. After half an hour the suspension was centrifugalized and the opalescent supernatant fluid was pipetted off. Two c.c. portions were pipetted into flasks containing 25 c.c. of 8% saccharose, 5 c.c. of $^{M}/_{15}$ potassium dihydrogen phosphate and 0.2 gram of colloid. In the cases of gelatinous aluminium hydrate and ferric phosphate 3 c.c. of a 3% suspension were used. Previous to the addition of the invertase, the saccharine fluids with their colloids had been brought to a pH value of 4.4 (pale green with brom-cresol green) with decinormal hydrochloric acid. Some of the colloids absorbed the acid, but the absorption had slowed down by the end of half an hour.

The flasks were then placed in a thermostat at 40° for 45 minutes, treated with 2 c.c. of basic lead acetate, cooled, made to 100 c.c. and filtered; 50 c.c. of the filtrate were treated with 5 c.c. of 10% potassium oxalate, acidified with dilute acetic acid, made to 200 c.c. and filtered. The invert sugar was determined in the filtrate by means of Fehling's solution. About 50% of the sugar was inverted under the conditions.

Preliminary tests showed some variation, but when the method as described was followed it was shown that the colloids had no influence upon the inversion of saccharose. The tests were made in three lots in order to avoid delay between the time of inversion and the determination of the invert sugar.

Table viii.—The Influence of Colloids upon the Inversion of Saccharose by Invertase. Percentage inverted.

Set 1.		Set 2.		Set 3.	
No colloid	53.1	No colloid	47.4	No colloid	49.0
Kaolin	52.7	French chalk ..	47.9	Silica	48.7
Kieselguhr	53.3	Charcoal	47.2	Talc	48.7
Diatomaceous earth	53.1	Asbestos	46.0	Ferric phosphate ..	47.4
Gelatin	53.4			Aluminium hydrate	44.9
Agar powder ..	53.4				

If the individual tests are taken to whole numbers, it will be clearly seen that the colloids were inactive. The low number for the gelatinous aluminium hydrate was probably due to the solution of the hydrate in the hydrochloric acid of which a considerable amount had to be added to bring the solution to pH 4.4.

The indifference of charcoal is in agreement with the work of Griffen and Nelson (*Journ. Amer. Chem. Soc.*, 1916, 722), who found that charcoal, alumina and albumen had no influence upon the activity of invertase.

Since the colloids do not accelerate the inversion of saccharose by invertase itself, it follows that the adsorption of the enzyme, if it does occur, is not the explanation of the efficiency of the colloids with living cells. The matter being of some importance, it was decided to test another enzymic fermentation, that of the decomposition of urea by bacteria and by the enzyme, urease.

The Ammoniacal Fermentation of Urea.

A flask with urea had undergone fermentation and from it a culture of *B. fluorescens liquefaciens* was obtained. It was seeded into flasks containing urea and buffer salts with and without kieselguhr. Six flasks were used, one for each determination of the ammonia by the method which will be described later.

Table ix.—The Fermentation of Urea by *B. fluorescens liquefaciens*. Percentage of urea fermented.

Days	1	2	4
Control	16.0	18.1	20.6
Kieselguhr	16.3	28.6	43.6

The kieselguhr undoubtedly accelerated the fermentation.

A urea-fermenting organism was isolated from soil. Morphologically it was like *Urobacillus duclauxii* and culturally like *U. pasteurii*.* It grew well at 37° on nutrient agar containing urea.

The bacterium was distributed in dilute meat-extract and an equal number of drops were seeded into twelve flasks, each containing 50 c.c. of a fluid consisting of 1% peptone, 1% urea and 0.5% sodium chloride. The urea was sterilized apart from the other components of the medium. The flasks were in groups of three, each group containing 0.2 gram of suspended colloid, with the exception of one group, which acted as control.

The results of the experiment show that these suspended colloids undoubtedly accelerated the activity of the bacterium.

Using the same organism, tests were made with other colloidal substances. The progress of the fermentation was watched both by the rise in the pH values and by the amount of ammonia found in a special control flask. This was found to be necessary when a progressive determination similar to the previous table was not made; one could not guess just when to stop the fermentation in order to show the effects of the colloids. In the two tests about to be tabulated all the flasks, with the exception of the sterile controls, contained bacteria. The infected

Table x.—The Influence of Colloids upon the Bacterial Fermentation of Urea. Percentage of urea fermented.

Days	1	2	3
Control	9.6	8.5†	14.5
Asbestos	15.5	51.7	95.6
Kaolin	15.2	31.2	96.0
Charcoal	11.4	20.2	39.0

* A motile rod with rounded ends. 0.6 : 2-3μ with longer and thread forms; spores were terminal and the Gram stain was positive. No growth was obtained on agar or gelatin devoid of urea, although, in bouillon without urea, a slight turbidity developed. In urea bouillon, a strong turbidity was produced. Milk without urea was unaffected. On nutrient agar with urea there developed at 37° a moist glistening translucent expansion. The colonies in urea gelatin appeared as a collection of pale brown lobules radiating from a central mass of small brown granules. These granules also appeared in the medium around the colony. In time the surface colonies became a mass of brown granules (not crystals) in a crateriform apparently liquefied area. The stab in urea gelatin was filiform surrounded by a white haze with little or no surface growth; at a later date (11 days) the gelatin was consumed, leaving a gas bubble, the upper part of the canal was ciliate and the lower part was studded with crystals, a haze was spread through the upper two-thirds of the gelatin.

According to Geilinger (through *Abst. in Bact.* iv, p. 93) the identification of the urea-fermenting bacteria is a matter of some difficulty.

† A large loop of the control of the second day was found to be sterile.

controls of set *a* were bright but they yielded growths of bacteria when sown upon urea-agar slopes and they had the faecal smell characteristic of the bacillus. Set *a* was stopped on the fourth day and set *b* on the third day. As in all previous cases, each flask in each set received the same number of drops of a well distributed culture of the bacillus. A sterile flask was included in each test in order to control the urea decomposed during the sterilization of the media. In set *a* this was equivalent to 4.1 and in set *b* to 4.6% of urea. These percentages have been subtracted from those obtained with the others.

The colloidal solutions of silicic acid and of ferric hydroxide coagulated upon being added to the saline medium; they acted, therefore, as insoluble colloids. The fluids were coloured with phenol-red and all were brought to the same pH value (7.2) before adding the sterile solution of urea.

Table xi.—The Influence of Colloids upon the Bacterial Fermentation of Urea. Percentage of urea fermented.

Set.		*a.*	*b.*
Silicic acid	0.1 gram	63.2	45.9
Silica	0.2 „	34.5	44.4
Aluminium hydrate	0.1 „	5.4	23.0
Aluminium phosphate	0.1 „	48.6	36.2
Ferric hydroxide	0.05 „	13.1	30.2
Kieselguhr	0.2 „	16.2	30.9
Fuller's earth	0.2 „	35.9	36.8
Asbestos	0.2 „	44.8	12.4
French chalk	0.2 „	7.6	11.7
Agar	0.2 „	6.9	5.4
Gelatin	0.2 „	3.6	11.0
Control (no colloid)		0.6 0.4	8.5 6.3

As a rule the colloids accelerated the fermentation of urea by the bacillus, and although they do not run parallel in each set their action is quite clear. The finely divided precipitate of silica formed on the addition of the silicic acid sol to the culture fluid was the most efficient accelerator.

During the sterilization of the fluids, the agar had softened and had set as a cake at the bottom of the flasks. Before infection the cake was chopped up with a platinum chisel; it acted as lumps of solid agar. After noting the activity of the finely divided precipitate of silica in the silicic acid test it was decided to try the effect of having the agar as a finely divided flocculent solid. Seven flasks were prepared as in the previous tests and in two of them the agar (= 0.2 gram) was shaken up after each sterilization. On cooling it separated in fine floccules. The same weight of agar-fibre was sterilized in test tubes without water and added to their respective flasks just before inoculation. One of the flasks served as a sterile control and gave an amount of ammonia equal to 5.0% of urea; this has been deducted from the other totals.

Table xii.—The Action of Agar in the Bacterial Fermentation of Urea. Percentage of urea fermented in five days at 37°.

Agar-fibre	16.4 16.9	16.6
Finely divided agar	20.9 21.2	21.0
Control (no agar)	9.1 8.3	8.7

The effect of the subdivision of the colloid is decided, but the difference is not so great as one might have expected. Similarly in set *b* of the previous table, the difference between the finely divided silica and the precipitated silica of commerce is not very pronounced.

The case having been proved for the fermentation of urea by bacteria, the influence of colloids upon urease was tested. Soy beans were used as the source of the enzyme. They were ground to a coarse meal and extracted with petrol. The defatted meal was ground to a fine flour and bottled for use. Two grams of the flour were shaken with 100 c.c. of water in a bottle at intervals during half an hour and filtered. Half a gram of urea was dissolved in 250 c.c. of water and 25 c.c. portions were pipetted into 100 c.c. conical flasks. These were fitted with corks, through each of which passed a rather wide tube holding a small plug of cotton-wool. The plug was subsequently moistened with dilute sulphuric acid and served to trap any ammonia attempting to escape from the flask. Each flask then received 10 c.c. of a buffer mixture of salts of pH value 5.6 and enough methyl-red to colour the fluid. The colloid, 0.2 gram, was added and the flasks were inserted in the thermostat at 40° where they remained for 15 minutes. They were examined and brought to a pH value of 5.6, that is, to a pale brick-red colour by the addition of a few drops of acid or alkali. Two c.c. of the soy bean extract (= 0.04 gram bean-flour) were added to each flask, the plugs were moistened with dilute acid and the flasks returned to the thermostat* where they remained for half an hour.†

The flasks were withdrawn from the thermostat, 5 c.c. of $^{N}/_{1}$ sulphuric acid were added to each, the plugs were pushed in and the tubes were washed into the flasks. The fluids were transferred to 500 c.c. flasks and boiled for 5 minutes to expel the carbon dioxide. After cooling, 1 gram of magnesium oxide was added and the flasks were connected up with condensers and boiled.‡ The distillates

Table xiii.—The Influence of some Colloids upon the Fermentation of Urea by Urease. Percentage of urea fermented.

Set.	1	2	3	4
Control (no colloid)	56	51	55	57
Asbestos	58	51	—	53
Silica	56	—	52	—
Kieselguhr	—	51	—	59
Talc	—	46	51	—
Fuller's earth	—	—	52	50
Charcoal	—	—	56	50
Ferric phosphate	—	—	—	56

* The thermostat consisted of a copper vessel with wires soldered vertically round the top. Each wire slid through the helical spring of an American clothes-peg which gripped the tube passing through cork of the flask. Thus the flasks were safely held while immersed in the water.

† The fluid lost its acid reaction as the ammonium carbonate was formed. A repeated adjustment to pH 5.6 had the effect of slowing the fermentation and was not adopted.

‡ This is a departure from the usual method which consists in adding a definite number of c.c. of standard acid to the fermented urea solution, eliminating the carbon dioxide by passing a current of air through the acidified solution and determining the free acid in the flask. It was found that the colloids absorbed more or less acid, sometimes as much as 1 c.c. (= 6% of urea fermented) and this rendered the method untrustworthy.

were caught in 20 c.c. portions of deci-normal acid and titrated, using Congo-red as the indicator. Controls showed that an allowance had to be made for the magnesium oxide.

The results showed that the controls were generally better fermented than the colloid tests and enough seemed to have been done to indicate that these substances do not accelerate the enzymic fermentation.

In the hope that some further information might be given by a slower fermentation, three tests were made in which the ammonia was determined at half-hourly intervals and in which a variable amount of bean extract was used.

Table xiv.—Varying Amounts of Urease. Percentage of urea fermented.

Ratio of Soy-bean meal to urea.		½ hour.	1 hour.	1½ hours at 40°.
4 : 5	Control	56	100	100
	Kieselguhr	51	91	100
2 : 5	Control	33	61	75
	Kieselguhr	27	56	71
3 : 5	Control	47	77	91
	Asbestos	46	76	91

The graphs of these numbers did not give any further information and the tests simply confirmed the previous work in showing that these colloids, rather than accelerate the fermentation, tended to retard it.

A very weak solution of urease was used in the following sequel to the last experiment.

Table xv.—The Slow Fermentation of Urea by Urease. Percentage fermented.

Days	1	2	3	4
a. Control	18.6	27.9	66.4	66.4
Kieselguhr ..	2.0	5.9	7.9	10.2
b. Control	23.5	40.9	55.9	55.9
Kaolin	17.4	28.6	38.1	40.6

Ratio of Soy-bean meal to urea, 1 : 25.

The slow fermentation exhibits in a more marked manner the inhibiting action of the colloid upon the urease.

We have thus come to the conclusion that the mineral colloids and suspensoids, such as have been used in the tests, assist the fermentation by living organisms such as bacteria and yeasts and depress or tend to depress the fermentation by the naked enzymes, invertase and urease.

Their depressing influence upon the enzymes is doubtless bound up with their faculty of being adsorbed upon the colloid and thus being removed from the mass of the substrate in the supernatant fluid.* The reason for their

* Waksman (*Abstracts of Bacteriology*, vi, 269) in a review of the literature upon the "Enzymes of Microorganisms," writes that enzymes "are readily taken up by finely divided substances such as kaolin, charcoal, infusorial earth, alumina, protein. . . . Adsorbed enzymes are inactive."

accelerating action upon the living cell cannot be explained and it seems reasonable to suppose that since the action is the opposite the *modus operandi* will not be on account of adsorption.

The Lactic Fermentation of Dextrose.

The lactic fermentation of dextrose promised to give further information regarding the influence of the colloids and a set of tests was prepared. The organism used was an acid-former that had been isolated from a liquid suspension of yeasts and bacteria sold by some local firms to bakers for raising their doughs. The preparations are known as spontaneous or "spon" yeasts. The bacterial flora is mixed, but by far the most numerous organism is one which grows feebly on the ordinary solid media, better on media containing dextrose and better still in dextrose nutritive fluids. Several of what were probably races of one organism were isolated; one* of them produced most acid in a modified Hansen's fluid, attaining a pH value of 3.4 as against 3.9 and 4.6 with the others.

The fluid in which the bacterium was grown contained starch glucose 5%, peptone 1%, potassium phosphate 0.35%, sodium phosphate 0.15% and sodium chloride 0.15%. The pH value was 6.6. Fifty c.c. were put into flasks together with 0.25 gram of the colloid except in the case of agar, of which 0.1 gram was taken. The agar liquefied during the sterilization of the flasks, and when the liquid cooled the agar settled out as a lumpy kind of clot.

During the experiment, 5 c.c. of fluid were extracted daily from each flask, coloured with a drop of phenolphthalein and titrated with $^{N}/_{20}$ potassium hydrate until a faint pink colour was obtained. The pink colour disappeared and after an interval of one minute the colour was adjusted with a further addition of alkali. In the experiments with urea, it had been found that the colloids absorbed hydrochloric acid, but in this experiment there appeared to be no absorption of the lactic acid formed by the microbe.

Two experiments were made, the first with a medium containing 0.5% of peptone but as the results were relatively the same as with a medium containing

Table xvi.—The Influence of Colloids upon the Lactic Fermentation of Dextrose. Acidity as c.c. of normal acid per litre of medium.

Time in days	2	3	4	5	6
French chalk	38	58	64	65	68
Talc	39	58	62	63	65
Animal charcoal	38	56	62	63	64
Willow charcoal	36	56	63	65	66
Asbestos	36	53	61	63	63
Fuller's earth	34	51	59	64	65
Kieselguhr	38	53	59	61	64
Silica	37	51	57	61	63
Kaolin	36	51	55	58	63
Control (no colloid)	37	49	56	60	63
Control (no colloid)	36	50	55	59	61
Gelatin	36	48	55	59	63
Agar	32	43	53	59	62

* It is a short rod, almost a coccobacterium, forming small translucent white colonies on dextrose agar. After some subcultivation, it grew rather better, especially on agar containing saccharose and glycerin. Dextrose-agar coloured with brom-cresol purple was rapidly turned yellow, even with the feebly growing cells. Milk was in some instances slightly acidified but never curdled. The acid formed in fluid dextrose media was almost entirely lactic. It grew better at 28° than at 37°.

1%, the second experiment is alone recorded. The figures for the fourth day of the 0.5% peptone test were practically the same as those of the third day with the 1%; the smaller amount of peptone reduced the acidity by about 8%.

The experiment shows that many of the colloids have accelerated the acidification of the fluid. French chalk, talc, the charcoals, asbestos and fuller's earth were the most efficient, while gelatin and agar had no action or depressed the fermentation. The general outcome is that the lactic fermentation falls into line with the other bacterial fermentations which are assisted by the presence of these colloids.

The Influence of Colloids on Diastatic Activity.

The action of malt-diastase upon starch was tested to see if it differed from urease and invertase in its behaviour in the presence of mineral colloids. Preliminary tests with iodine as an indicator showed that the qualitative method was not sufficiently regular in its action to bring forward the results as evidence of the activity or otherwise of the colloids. The actual determination of the sugar resulting from the fermentation promised to be more acceptable.

Two grams of soluble starch were put into a 100 c.c. conical flask along with 50 c.c. of water. The starch was liquefied and upon cooling 5 c.c. of a buffer mixture of salts of pH value 4.9 were added. Two grams of ground malt were digested overnight at 22° in 100 c.c. of water. The clear filtrate was diluted fourfold and 5 c.c. were pipetted into each flask together with 0.2 c.c. of chloroform. The flasks were corked and incubated overnight at 37°. The slow fermentation with the small quantity of malt extract (= 0.025 gram of malt) converted about 60% of the starch and this was considered to be about the extent of change best suited to show the effect of the added colloid, of which 0.2 gram was present in each flask.

Some tests with a larger proportion of malt had given from 75% to 78% of altered starch, but as this is so near the point where starch shows a resting stage or rather a slowing up in the fermentation (*Trans. Guinness Research Lab.* i (1), 87), the smaller quantity was used.

Table xvii.—The Influence of Certain Colloids upon the Saccharification of Starch by Diastase. Maltose from 100 grams of dry starch.

Test	1	2	3
Control	63.2	61.5	64.1
Kieselguhr	25.3	36.9	—
Silica	42.6	50.0	—
Talc	48.1	59.7	50.9
Charcoal	55.4	56.0	—
Asbestos	57.9	49.9	57.9
French chalk	16.7	53.1	31.5
Kieselguhr (local)	9.4	15.6	—
Kaolin	8.0	33.3	9.5
Fuller's earth	9.2	8.8	—
Agar powder	58.7	53.1	—

The results show that the mineral colloids with charcoal and agar not only did not assist the diastase to saccharify the starch, but also that they varied in their activity in depressing the saccharification. This point has been noted, to some extent, by other investigators in regard to certain mineral and organic

colloids. Pincussen (*Bot. Abstracts*, 1924, 692) reported that a number of enzymes including the diastases were depressed by colloidal ferric hydroxide, and Hagihara noted that cholesterol depressed diastatic action.

It is possible that if the enzyme had been allowed to act more slowly, the colloids might have had time to exert some influence.

To test this point, a weaker extract of malt was taken and the tests were incubated at a lower temperature, viz. 37°. The 2% solution of malt was diluted ten times, so that the ratio of malt to starch was 0.5 to 100 as against 1.25 to 100 in the previous table. Twelve flasks were used in the experiment, four controls and four each with asbestos and animal charcoal.

Table xviii.—The Slower Fermentation of Starch by Diastase.
Maltose from 100 grams of dry starch.

Days	1	2	3	4
Control	52.3	57.3	62.2	61.0
Charcoal	41.6	48.6	58.9	56.3
Asbestos	37.5	50.2	51.6	56.1

The results are similar to those already obtained and show that, whether by a slow or by a quick fermentation, the colloids such as charcoal and asbestos lessen the activity of the enzyme.

The Influence of Colloids upon the Diastatic Activity of Starch-dissolving Bacteria.

Raw starch contains a number of starch-dissolving bacteria among which are *Bac. mycoides** and *Bac. vulgatus*. The latter is by far the most numerous and a colony was selected for testing the diastatic activity. It turned out to be *Bac. levaniformans*, an ally or race of *Bac. vulgatus*, that produces an opalescent solution of gum-levan when grown in fluids containing saccharose, peptone, sodium phosphate and potassium chloride. The bacillus was grown in a nutrient fluid consisting of 1% of meat extract with 0.15% of potassium phosphate and brought to a pH value of 6.8. Kaolin and asbestos were chosen for testing the hydrolytic effect. As in most of the previous experiments, each test had a flask to itself; there were twelve flasks each containing 50 c.c. of fluid, 2 grams of soluble starch and 0.25 gram of colloid except in the case of the controls.

Although the organism is an active starch dissolver, it did not promise to show well in the experiment for the reason that in bouillon and in meat extract

Table xix.—The Action of *Bac. levaniformans* upon Soluble Starch.
Maltose from 100 parts of dry starch.

Days	2	3	4	5
Control	48.5	59.5	62.6	69.0
Kaolin	50.1	64.1	67.2	69.3
Asbestos	48.0	59.1	64.9	68.5

* *Bac. mycoides* and another starch-dissolving bacterium, both isolated from starch, were examined, but they did not seem to be capable of converting soluble starch to maltose. The products of fermentation, after clarification, did not reduce Fehling's solution in a normal manner like maltose. During the boiling the solution became blackish and suddenly turned to a yellow-green colour.

it forms a strong cohesive film on the surface of the fluid, with very little sub-surface growth. If contact between the colloid and the bacterium is a desideratum, it would not occur to any extent with this bacterium. The results were according to promise and the colloids tested had little or no influence. Kaolin did accelerate a little but asbestos did not.

At a later date the matter was again tested with another culture of the same organism isolated from sugar. Each test flask contained 50 c.c. of a medium consisting of meat extract 0.5%, monopotassium phosphate 0.15% and sodium chloride 0.5% brought to a pH value of 6.5. Each flask also contained 2 grams of soluble starch and, excepting the controls, 0.25 gram of colloid. The fermented fluid was transferred to a 200 c.c. graduated flask with the aid of 50 c.c. of water, 6 c.c. of basic lead acetate were added and then strong spirit to volume. After shaking, cooling and adjusting to volume, the fluid was filtered into a graduated jar and a known volume of the filtrate was transferred to a basin, treated with 10 c.c. of 10% potassium oxalate and 0.5 c.c. of dilute acetic acid and evaporated until the spirit had been expelled. The residual fluid was made to 200 c.c. and filtered. The reducing sugar in the filtrate was determined as maltose.* The treatment of the fermented starch with basic lead acetate and spirit was distinctly advantageous in obtaining a clear filtrate.

Table xix*a*.—The action of *Bac. levaniformans* upon Soluble Starch. Maltose from 100 parts of dry starch.

Days	2	3	4	5
Control	16.5	56.4	58.5	55.4
Animal charcoal	19.5	60.2	59.2	59.4
Fuller's earth	31.3	60.8	62.9	59.6
Silica	19.8	56.6	58.8	55.6
Asbestos	17.9	56.9	55.9	53.1

It is evident that the mineral colloids and charcoal have a distinct influence in accelerating the diastatic activity of the bacterium more especially in the early stages of the fermentation. The most indifferent was asbestos which, as in Table xix, had little or no action. Animal charcoal and fuller's earth were the most efficient accelerators.

A test was made to see the action of talc upon the diastatic activity of *Bac. levaniformans*. Two flasks, each containing a litre of medium similar to that

Table xix*b*.—The Influence of Talc in accelerating the diastatic Activity of *Bac. levaniformans*. Maltose from 100 grams of dry starch.

Days	3	4	7	11
Control	42.3	55.6	50.5	38.7
Talc	46.9	55.8	51.3	40.7

* *Bac. vulgatus* is known to produce diastase. Vignal found that it also produced invertase, protease, cytase and rennin. Effront noted that its diastatic enzyme had strong amylolytic and weak saccharifying powers (Waksman, Enzymes of Micro-organisms, *Abstr. of Bact.* vi (1922), 281). Estienne (through *Journ. Soc. Chem. Ind.*, 1925, B184) notes that it also secretes maltase. Judged by the reducing powers of a solution before and after boiling with dilute hydrochloric acid, the sugar formed by the action of *Bac. levaniformans* upon starch was maltose.

used in Experiment xix, were seeded with the bacillus. One flask received 5 grams of gently ignited talc. Portions measuring 100 c.c. were abstracted from each flask from time to time and treated as in Experiment xix*a*.

As with the colloids of Experiment xix*a*, talc shows an accelerating action in the early stages of the fermentation. In addition, it shows that in course of time the maltose becomes consumed and the colloid tends to inhibit this consumption. It is possible that there may be a balance between the formation and the destruction of the sugar and that the impetus given to the formation by the talc makes it appear as if it inhibited the destruction.

The experiments tend to show that the diastatic activity of bacteria, as exemplified by this form of *Bac. vulgatus*, is accelerated by the presence of some mineral colloids and of charcoal. The evidence is not so pronounced as in other cases, but this may possibly be due to the unsuitability of the organism. It may also be due to the fact that starch itself is a colloid and the effect of the additional colloid cannot be so pronounced as when a non-colloid substrate is employed.

Conclusion.

Certain mineral and other colloids were tested to see if they accelerated the fermentation of carbohydrates by yeast after the manner that they hastened the fermentation of certain compounds of carbon by the high temperature organism. Silica induced a quicker production of carbon dioxide in the fermentation of saccharose by a distillery yeast. Asbestos and silica hastened the inversion of saccharose by yeast and silica accelerated the fermentation of dextrose. Wood-charcoal, talc, kaolin, fuller's earth, asbestos, kieselguhr, French chalk and ferric hydrate also hastened the slow fermentation of dextrose. The differences in the relative amounts of fermented material were not so great as when dealing with bacteria, but they were sufficiently marked to show that the yeasts and the bacteria obeyed a rule and were favourably influenced by the presence of these colloidal substances.

The inversion of saccharose by *Bac. levaniformans*, an organism closely related to and perhaps identical with *Bac. vulgatus*, was hastened by the presence of mineral and other colloids, as silica, fuller's earth, French chalk, kieselguhr, asbestos, kaolin, talc and charcoal. The inversion of the sugar by diastase, prepared from yeast, was not influenced by these colloids.

The ammoniacal fermentation of urea by *Bac. fluorescens liquefaciens* was assisted by the presence of kieselguhr. The fermentation of urea by a urobacillus was accelerated by most of the previously named colloids and by agar, gelatin, silicic acid, aluminium hydrate and aluminium phosphate. Agar in floccules was more effective than in the fibrous condition.

These colloids had no influence upon the fermentation of urea by the enzyme urease contained in an extract from the soy bean, and it did not matter whether the fermentation was quick or slow.

The lactic fermentation of dextrose by a lactic bacterium was hastened by most of these colloids; kaolin, gelatin and agar did not act.

The diastatic fermentation of starch by *Bac. levaniformans* was accelerated by kaolin, animal charcoal, fuller's earth and talc. The acceleration was not pronounced, possibly because starch is itself a colloid, but it was sufficient to show that the bacterial fermentation of starch falls into line with other fermentations.

The colloids had an inhibiting influence upon the action of malt diastase on starch.

It is evident that the influence of the insoluble colloids in accelerating the fermentative activities of bacteria and yeasts is not due to the adsorption of their enzymes.

All the colloids were insoluble. When the sols of silicic acid and ferric hydroxide were used, they were coagulated by the salts of the media necessary for the growth of the microorganisms, and therefore acted as insoluble colloids. The soluble colloid, gelatin, feebly stimulated the urobacillus, depressed the alcoholic and had no action upon the acid fermentation of dextrose.

The research has shown that yeasts and bacteria have their fermentative activities accelerated by the presence of certain mineral and other colloids such as talc, kieselguhr, silica, fuller's earth, charcoal and agar and that the isolated enzymes are not influenced by the same colloids.

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